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c-di-AMP Reports DNA Integrity during Sporulation in B. subtilis

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Referee Reports

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The mansucript of Oppenheimer-Shaanan et al describes an investigation of DisA and c-di-AMP related signaling in B.subtilis and provides the first in vivo study for this newly identified bacterial nucleotide. The authors originally identified DisA as novel bacterial sporulation checkpoint protein and DisA later turned out to be a synthetase for a previously undescribed prokaryotic nucleotide, c-di-AMP. In this manuscript, the authors provide evidence that c-di-AMP, synthesized by DisA, is indeed a second messenger. For instance, the sporulation is delayed upon switching off the DisA activity or increasing the Ybt-phosphodiesterase expression. Using externally supplied c-di-AMP the authors can recover the sporulation properties of deltaDisA-cells and show that external c-di-AMP supply overrides the sporulation inhibition in presence of DNA harming substances. All an all, this is an important validation of c-di-AMP in living cells and its second messenger function which I believe is relevant for a broad audience. Most of the manuscript is well written but there are some overstatements that should be more carefully rewritten:

1. It is not exactly clear from which experiment the authors conclude that DisA "actively" scans the chromosomes, rather than by passive diffusion. While the structural results argue against an active scanning mechanism, the initial demonstration of active scanning by more or less killing the cells with azide in the original DisA paper of the Ben-Yehuda lab is in my opinion no evidence for active scanning.

2. Along the same lines, the authors do not provide any experimental support that DisA directly binds to ds-breaks. As far as I know the exact pathways of nalidixic acid in inducing SOS response leading to ds-breaks are not yet entirely clear. Thus DisA might also be recognizing intermediate structures occurring after nalidixic acid induced inhibition of the gyrase cycle. The authors should provide data that DisA actually binds the breaks and no colocalize during ongoing repair by recognizing something else.

3. The authors describe that the DisA D77N mutant does not form foci. Is the construct somehow deviating from the GFP-DisA wildtype Protein in addition to D77N, e.g by an additional tag? Also, it would be interesting to see whether focus formation can be restored by supplying external c-di-AMP.

4. There should at least be an additional schematic supplemental detailed picture of the constructs used in all of the experiments, the fact that table S1 gives information like e.g. MB3: "disA-gfp-spc" is a bit unclear.

5. Fig 2A: please indicate the c-di-AMP peak (7.15ml). Which molecule is represented by the much larger peaks at ~7.5ml ? Is there any MS-data for that?

Referee #2 (Remarks to the Author):

This manuscript is based on the published finding that DisA in B. subtilis synthesizes c-di-AMP and the authors' previous findings that DisA is a checkpoint protein during sporulation. Here they demonstrate that the c-di-AMP level increases during sporulation in a DisA-dependent manner and that DNA damage dicreases the c-di-AMP level. They also show evidence that the YybT protein is responsible for degrading c-di-AMP and part of the DisA-dependent checkpoint.
The work is well done and the data support the conclusions. I guess it could be said that the data are not exactly surprising, but it is good to know that the presumed activity of DisA is what determines its checkpoint activity. No indication of a target or a mechanism is indicated, so I am not convinced that this represents a major advance.
The ms maybe longer than necessary and I am not sure that all the micrographs of cells are necessary. The ms. is a bit imprecise here and there (e.g. .."we investigated how DAC activity affecs DisA function in vivo"), and in particular the conclusions are sometimes stretched a bit. I guess they have not conclusively shown that c-di-AMP is the signalling molecule for the checkpoint, although it seems highly likely.
Some specific comments:
The way the concentration(s) of c-di-AMP is given (Fig 2 and text) seem to suggest that this micromolar range is the intracellular range. The way I understand the assay says that this concentration is simply the one that comes out after the dilutions and treatments that are done in this assay. Thus, it is ony the relative levels that are important.
Fig. 6 is not particularly helpful.

1st Revision - authors' response

Response to the referees:

Referee #1 (Remarks to the Author):

1. It is not exactly clear from which experiment the authors conclude that DisA "actively" scans the chromosomes, rather than by passive diffusion. While the structural results argue against an active scanning mechanism, the initial demonstration of active scanning by more or less killing the cells with azide in the original DisA paper of the Ben-Yehuda lab is in my opinion no evidence for active scanning.

Since this point has very little to do with the current paper, the word "actively" was omitted.

2. Along the same lines, the authors do not provide any experimental support that DisA directly binds to ds-breaks. As far as I know the exact pathways of nalidixic acid in inducing SOS response leading to ds-breaks are not yet entirely clear. Thus DisA might also be recognizing intermediate structures occurring after nalidixic acid induced inhibition of the gyrase cycle. The authors should provide data that DisA actually binds the breaks and no colocalize during ongoing repair by recognizing something else.

In the current paper the majority of the experiments were carried out using two different DNA damaging agents MMC and nalidixic acid that efficiently activate the DisA-checkpoint response. Thus, we think it is less likely that DisA recognizes a structure specific to the nalidixic acid pathway.
In addition, we have demonstrated in our previous publication (Bejerano-Sagie et al., 2006) that DisA binds to DSBs. In brief, to show that DisA binds DSBs, we introduced the yeast HO endonuclease and its recognition sequence to the genome of B. subtilis. This enzyme is known to produce DSB by cleaving its recognition sequence. We have demonstrated using two independent methods that upon induction of the endonuclease, DisA binds specifically to the break site:
1) Colocalization experiments: Fluorescence in situ hybridization (FISH) (to visualize the DNA break site) in conjunction with immunofluorescence (to visualize DisA)
2) Chromatin Immunoprecipitation (ChIP).
These data are presented in Figure 5 (Bejerano-Sagie et al., 2006).
Consistently, DisA binds to branched-DNA in vitro, a structure that mimics DSB repair intermediates (Witte et al, 2008).

We cannot exclude the possibility that DisA recognizes several DNA structures or repair components, however, we have no evidence for such activity and that matter will be the focus of future study.

3. The authors describe that the DisA D77N mutant does not form foci. Is the construct somehow deviating from the GFP-DisA wildtype Protein in addition to D77N, e.g by an additional tag? Also, it would be interesting to see whether focus formation can be restored by supplying external c-di-AMP.

This experiment was described in our original submission and probably missed by the reviewer. In the current version we emphasized the outcome of this experiment p8 2nd paragraph).
"Supplementation of exogenous c-di-AMP promoted sporulation but failed to restore formation of the DisA focus (data not shown), suggesting that the molecule has to be synthesized by the protein to maintain the focal structure."

4. There should at least be an additional schematic supplemental detailed picture of the constructs used in all of the experiments, the fact that table S1 gives information like e.g. MB3: "disA-gfp-spc" is a bit unclear.

Following the reviewer's comment we reorganized the plasmid construction section attempting to make it clearer. In addition, plasmid genotypes were added to Table S1 as part of the strains' description. Some of the B. subtilis strains were described in details in our previous publications, for these strains only genotypes and references are given in Table S1.

5. Fig 2A: please indicate the c-di-AMP peak (7.15ml). Which molecule is represented by the much larger peaks at ~7.5ml ? Is there any MS-data for that?

We did not analyze the peak adjacent to c-di-AMP. We focused on the peak with a retention time identical to that of purified c-di-AMP and explored it further by MS analysis. In addition, we could not detect any difference within the large pick between extracts from the wild-type and the disA cells.

References
1) Bejerano-Sagie M, Oppenheimer-Shaanan Y, Berlatzky I, Rouvinski A, Meyerovich M, Ben-Yehuda S. 2006. A checkpoint protein that scans the chromosome for damage at the start of sporulation in Bacillus subtilis. Cell 125: 679-690.

2) Witte G, Hartung S, Buttner K, Hopfner KP. 2008. Structural biochemistry of a bacterial checkpoint protein reveals diadenylate cyclase activity regulated by DNA recombination intermediates. Mol Cell 30: 167-178.

Referee #2 (Remarks to the Author):

Some specific comments:
The way the concentration(s) of c-di-AMP is given (Fig 2 and text) seem to suggest that this micromolar range is the intracellular range. The way I understand the assay says that this concentration is simply the one that comes out after the dilutions and treatments that are done in this assay. Thus, it is ony the relative levels that are important.
Fig. 6 is not particularly helpful.

Regarding the c-di-AMP concentrations, we agree with the reviewer and we modified the main text and the figure legend accordingly to clarify this point.
Text: On page 7 (2nd paragraph) the word "determine" was replaced with "compare".
Figure-2, legend: The following sentence was added: "The concentrations of c-di-AMP were determined by suspending whole extracts in the same volume".

According to the reviewer's request and the editor's instructions the manuscript was significantly shortened. However, we do find previous Fig 6 to be helpful for summarizing our results and explaining our model. We resized the figure and included it as part of Figure 5 (Fig 5C).

Editorial Decision

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